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The single NqrB and NqrC subunits in the Na^+-translocating NADH: Quinone oxidoreductase (Na^+-NQR) from *Vibrio cholerae* each carry one covalently attached FMN☆

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ABSTRACT

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is the prototype of a novel class of flavoproteins carrying a riboflavin phosphate bound to serine or threonine by a phosphodiester bond to the ribityl side chain. This membrane-bound, respiratory complex also contains one non-covalently bound FAD, one non-covalently bound riboflavin, ubiquinone-8 and a [2Fe–2S] cluster. Here, we report the quantitative analysis of the full set of flavin cofactors in the Na^+-NQR and characterize the mode of linkage of the riboflavin phosphate to the membrane-bound NqrB and NqrC subunits. Release of the flavin by β-elimination and analysis of the cofactor demonstrates that the phosphate group is attached at the 5'-position of the ribityl as in authentic FMN and that the Na^+-NQR contains approximately 1.7 mol covalently bound FMN per mol non-covalently bound FAD. Therefore, each of the single NqrB and NqrC subunits in the Na^+-NQR carries a single FMN. Elimination of the phosphodiester bond yields a dehydro-2-aminobutyrate residue, which is modified with β-mercaptoethanol by Michael addition. Proteolytic digestion followed by mass determination of peptide fragments reveals exclusive modification of threonine residues, which carry FMN in the native enzyme. The described reactions allow quantification and localization of the covalently attached FMNs in the Na^+-NQR and in related proteins belonging to the *Rhodobacter* nitrogen fixation (RNF) family of enzymes. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

Flavoproteins are a diverse class of redox proteins involved in many biological processes [1]. Typically, their flavin coenzymes are non-covalently bound, but a subset of proteins is known, which comprise a covalent linkage of the flavin moiety to an amino acid side chain [2]. Based on the type of linkage, three classes of covalent flavoproteins have been identified: (i) FAD or FMN are linked at the 8α-methyl group to a Tyr, His or Cys residue; (ii) FMN is linked at the C6 atom of the isoalloxazine ring to a Cys residue; (iii) riboflavin phosphate forms a phosphodiester with a Thr or Ser residue. The latter mode of flavin modification is found in the Na^+ -translocating NADH:quinone oxidoreductase (Na^+-NQR), a redox driven ion pump present in many pathogenic and non-pathogenic bacteria, which represents a functional equivalent of respiratory Complex I. The Na^+-NQR transports Na^+ ions upon the oxidation of NADH by ubiquinone (Q). Its six subunits (NqrA-F) are not related to subunits of complex I and harbor a different set of cofactors: a non-covalently bound FAD and a 2Fe–2S cluster in the peripheral NqrF subunit, riboflavin phosphate, which is covalently bound to the peripheral NqrC subunit, another covalently bound riboflavin phosphate plus a non-covalently bound riboflavin found in the membranous NqrB subunit, and ubiquinone-8 in the peripheral NqrA subunit [3–9].

MS analysis of proteolytic digests of Na^+-NQR from *Vibrio alginolyticus* showed attachment of riboflavin phosphate to Thr residues of subunits NqrB and NqrC *via* a phosphodiester bond to the ribityl chain [5]. In *Vibrio sp.*, the modified Thr residue is part of a conserved Thr-Gly-Ala-Thr motif. Other proteins harboring this motif were shown to contain covalently bound flavin, such as RnfG and RnfD from *Vibrio cholerae*, two subunits of the Na^+-NQR related *Rhodobacter* nitrogen fixation (RNF) complex [10].

Abbreviations: Na^+-NQR, Na^+-translocating NADH:quinone oxidoreductase; RNF complex, *Rhodobacter* nitrogen fixation complex; Q, ubiquinone; DDM, *n*-dodecyl-β-D-maltoside; SVPD, snake venom phosphodiesterase; β-ME, β-mercaptoethanol

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Notably, in subunit RnfD, which is related to NqrB, the Thr residue within the conserved sequence stretch (T-G-A-T) was not modified [10], indicating that the attachment site for FMN in NQR and its homologs cannot be predicted from this amino acid sequence. Here we show that each of the single NqrB and NqrC subunit of the Na^+-NQR from *V. cholerae* carry one covalently attached riboflavin phosphate bound via a phosphodiester between Thr and the 5'-position of the ribityl side chain as in authentic FMN. The experimental strategies described herein are applicable for all members of the growing families of the NQR and the related RNF complexes, which play a prominent role in energy conservation in many bacteria.

2. Material and methods

2.1. Analytical methods

NADH, FAD, FMN and FMN were purchased from Sigma-Aldrich. Riboflavin was obtained from Fluka. FMN (4 mM in 50 mM Na^+-acetate buffer, pH 5.0) was further purified by HPLC on an EC 250/4 Nucleosil 120–5 C18 column [3,11]. Fractions collected from 27.8 to 29.0 min contained FMN. Fractions collected from 21.4 to 27.6 min contained FMN (riboflavin 5'-monophosphate) and riboflavin 4'-monophosphate at a ratio of approximately 1:1. The structures of the flavins are shown in Fig. 1. Protein was determined by the bicinchoninic acid method using the reagent from Pierce [12]. BSA (fraction V; from Applichem) served as standard.

2.2. Purification of Na^+-NQR

Na^+-NQR from *V. cholerae* was produced and purified as a recombinant protein containing a His_6-tag at the N-terminus of subunit NqrA [3]. Briefly, His_6-tagged Na^+-NQR was expressed in *V. cholerae*, solubilized with *n*-dodecyl-β-D-maltoside (DDM) and purified via nickel affinity chromatography. The enzyme was concentrated to 1–4 mg ml^{-1} in buffer containing 50 mM sodium phosphate, pH 8.0, 5% glycerol, 300 mM NaCl and 0.05% DDM and further purified by gel filtration [3].

2.3. Cleavage of FMN with snake venom phosphatase

Snake venom phosphodiesterase I (SVPD) type IV from *Crotalus atrox* venom was purchased from Sigma-Aldrich. Fifty microliters of Na^+-NQR (2.5 mg ml^{-1}) were mixed with 0–0.8% SDS (final concentration) using a 10% (w/v) SDS solution and incubated in the dark for 30 min under gentle shaking at 25 °C. By adding 2 ml buffer A (10 mM HEPES•NaOH, pH 8.0, 300 mM NaCl, 0.05% DDM, 5% glycerol) the concentration of SDS was reduced to less than 0.02% to maintain the enzymatic activity of SVPD [13]. Na^+-NQR was concentrated again to 0.1 ml using centrifugal filter devices (Ultrafree-MC, 5 kDa cut-off, Millipore). Two aliquots of 40 µl were withdrawn. One aliquot was mixed with 2 µl SVPD solution (3 units ml^{-1}), the other served as control. Both aliquots were incubated for 10 min at 25 °C, followed by analysis on SDS-PAGE. SDS-PAGE was performed with 10% polyacrylamide gels in the presence of 6 M urea [14,15]. The Na^+-NQR was allowed to react with sample buffer (50 mM Tris•HCl, pH 6.8, 5% SDS, 5.8% glycerol, and 0.1 M β-mercaptoethanol) for 30 min at room temperature to prevent precipitation of the hydrophobic NqrB, NqrD and NqrE subunits [15]. Thirty micrograms of protein were loaded per lane. Gels were analyzed for flavins with a fluorescence scanner (excitation, 457 nm; emission, 526 nm; Typhoon 9400 scanner, Molecular Dynamics). Proteins were stained with Coomassie brilliant blue G-250.

2.4. Quantification of FMN released from denatured Na^+-NQR under alkaline conditions

To determine the content of covalently bound FMN in the Na^+-NQR, it was necessary to remove the non-covalently bound flavins. Briefly, protein from a concentrated solution (1–4 mg ml^{-1}) was precipitated with 6.25% TCA and removed by centrifugation [3]. The supernatant containing the non-covalently bound flavins was neutralized by adding 0.8 M K_2HPO_4 and passed through PVDF filter (0.22 µm; Millipore) prior to analysis by HPLC. The protein pellet obtained by the initial TCA precipitation was further used to extract covalently bound FMN. The pellet was washed repeatedly with 6.25% TCA (0 °C), until the supernatants were free of non-covalently bound flavins as confirmed by HPLC.

To release the FMN, ice-cold LiOH (0.5 M, 0.1 ml) was added to the pellet, which was resuspended by vortexing and stored on ice for 24 h. At this step, protection of flavins from light was crucial to prevent their photolysis. Fifty microliters 30% TCA (0 °C) were added, shifting the pH to the acidic range and precipitating proteins. After an incubation of 5 min at 0 °C, the suspension was cleared by centrifugation (5 min, 15,800 × *g*). The supernatant containing the flavins released from subunits NqrB and NqrC was neutralized by adding 0.8 M K_2HPO_4 and passed through a PVDF filter (0.22 µm; Millipore) prior to analysis by HPLC [3]. Standard solutions for quantification and identification of flavins by HPLC contained 120 pmol FAD, 37 pmol FMN and 56 pmol riboflavin. Flavin concentrations were determined photometrically using the following extinction coefficients at 450 nm:

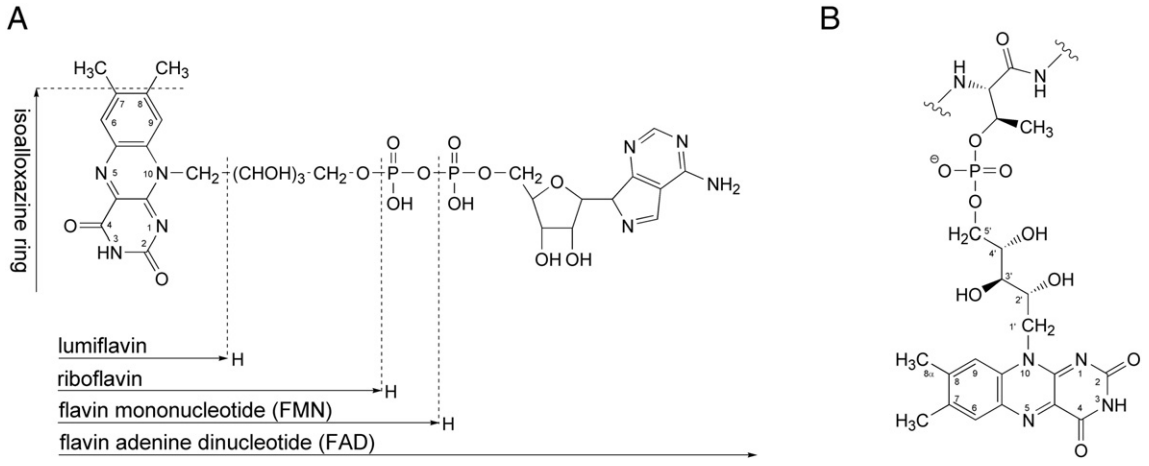

Fig. 1. Structure of common flavins and phosphodiester-linked FMN. Nomenclature of flavins (A) and structure of the phosphodiester-bound FMN (B), which is linked between the 5'-hydroxyl group of the ribityl chain and the hydroxyl group of a Thr side chain to NqrB and NqrC subunits of the Na^+-NQR.

FAD, 11.3 $mM^{-1}\,cm^{-1}$;[16] FMN, 12.5 $mM^{-1}\,cm^{-1}$, and riboflavin, 10.7 $mM^{-1}\,cm^{-1}$ [17]. FAD, FMN and riboflavin eluted from the HPLC column after 25.3, 27.3, and 29.3 min, respectively.

2.5. Identification of FMN-carrying amino acid residues

Fifty microliters of Na^+-NQR (approx. 10 mg ml^{-1}) in buffer A were mixed with 50 µl 1 M LiOH and 0.7 µl β-mercaptoethanol. After 27 h incubation at 0 °C in the dark, 40 µl of the five-fold concentrated SDS sample buffer without β-mercaptoethanol, 20 µl 2 M HCl, and 4 µl 1 M dithiothreitol were added. The following steps were carried out in the dark. After incubation for 20 min at room temperature, 25 µl iodoacetamide solution (1 M, final concentration 125 mM) was added, and the mixture was allowed to react for 20 min. By adding 14 µl 1 M dithiothreitol, excess iodoacetamide was quenched after 20 min. Proteins were separated by SDS-PAGE as described above. The protein bands corresponding to subunit NqrB and NqrC were excised for in-gel digestion. The excised gel bands were destained with 30% acetonitrile, shrunk with 100% acetonitrile, and dried in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with elastase were performed overnight at 37 °C in 50 mM NH_4HCO_3 (pH 8). About 0.1 µg of protease was used for one gel band. Peptides were extracted from the gel slices with 5% formic acid.

All LC-MS/MS analyses were performed on a Q-TOF mass spectrometer (Agilent 6520, Agilent Technologies) coupled to a 1200 Agilent nanoflow system *via* a HPLC-Chip cube ESI interface. Peptides were separated on a HPLC-Chip with an analytical column of 75 µm i.d. and 150 mm length and a 40-nl trap column, both packed with Zorbax 300SB C-18 (5 µm particle size). Peptides were eluted with a linear acetonitrile gradient with 1% min^{-1} at a flow rate of 300 nl min^{-1} (starting with 3% acetonitrile). The Q-TOF was operated in the 2 GHz extended dynamic range mode. MS/MS analyses were performed using data-dependent acquisition mode. After an MS scan (2 spectra s^{-1}), a maximum of three peptides were selected for MS/MS (2 spectra s^{-1}). Singly charged precursor ions were excluded from selection. Internal calibration was applied using two reference masses.

Mascot Distiller 2.3 (Matrix Science Ltd.) was used for raw data processing and for generating peak lists, essentially with standard settings for the Agilent Q-TOF. Mascot Server 2.3 was used for database searching with the following parameters: peptide mass tolerance: 20 ppm, MS/MS mass tolerance: 0.05 Da, enzyme: "none," variable modifications: Carbamidomethyl (C), Gln→pyroGlu (N-terminal Q), and oxidation (M), β-mercapto (ST). For protein and peptide identification a small custom database containing the protein sequences of NqrA, NqrB, and NqrC was used. All MS/MS spectra, which indicated β-mercapto-modification of peptides were validated manually.

3. Results and discussion

3.1. Release of covalently bound FMN from Na^+-NQR

The quantitative analysis of the full set of flavin cofactors of the Na^+-NQR is hampered by the hydrophobicity of the flavinylated NqrB and NqrC subunits. In principle, one would first determine the content of non-covalently bound flavins in the supernatant of the Na^+-NQR after precipitation with TCA. In a next step, the precipitated, flavinylated subunits would be treated with, e.g., snake venom phosphodiesterase (SVPD) to release the covalently attached flavins [18]. We separated the subunits of the Na^+-NQR by SDS-PAGE to detect flavins covalently attached to NqrB and NqrC from their intrinsic fluorescence (Fig. 2). The weaker signal observed for the NqrB subunit is most likely caused by quenching of the flavin fluorescence by a nearby tryptophan residue, which is absent in NqrC [19]. SVPD has been reported to hydrolyse various phosphodiesters [18] and also FAD, which is hydrolysed to FMN [13,20,21]. Treatment of the native Na^+-NQR with SVPD released no or only little amounts of covalently linked flavins from NqrB and NqrC (Fig. 2). Upon partial denaturation of Na^+-NQR in the presence of SDS, the phosphodiester bond linking the riboflavin phosphates to Thr residues of NqrB and NqrC became accessible for hydrolysis by SVPD. Increasing the SDS concentration to 0.8% resulted in complete release of riboflavin phosphates by SVPD (Fig. 2). An advantage of this enzymatic method for identification and release of phosphodiester-linked flavins is that digestion of proteins by proteases is not required and that simultaneous screening for phosphodiester-linked flavins in many specimens is possible. In principle, this method would also be suitable for quantification of bound flavin. However, in the Na^+-NQR, the situation is complicated by the presence of non-covalently bound FAD and riboflavin, which are expected to be at least partially released during denaturation of the complex by SDS. In case of the Na^+-NQR, removal of non-covalently bound FAD and riboflavin prior to analysis of covalently bound FMNs is therefore mandatory. This is usually achieved by complete denaturation of the Na^+-NQR and separation of precipitated proteins by centrifugation. However, precipitated subunits of the Na^+-NQR, especially the hydrophobic NqrB and NqrC subunits binding the FMN, cannot be solubilized under conditions maintaining the enzymatic activity of SVPD. Another disadvantage of the enzymatic method is that the SVPD which is commercially available as crude snake venom contains traces of FAD [22]. We therefore chose a non-enzymatic approach where the TCA precipitation step resulting in release of non-covalently bound FAD and riboflavin from subunits NqrF and NqrB [8] was followed by treatment of the precipitated, yellow NQR pellet with LiOH. Covalently attached flavins should be released by β-elimination under alkaline conditions, similar to citrate lyase, whose prosthetic group is bound via a phosphodiester to a Ser residue [23].

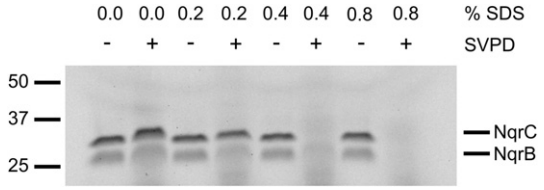

Fig. 2. Enzymatic release of FMN from subunits NqrB and NqrC of the Na^+-NQR. Covalently bound flavins on NqrB and NqrC were detected by recording in gel fluorescence after SDS-PAGE. Na^+-NQR was denatured with SDS at increasing concentrations as indicated (0–0.8%) and mixed with SVPD. Controls in the absence of SVPD were performed in parallel. Molecular mass of marker proteins (left) in kDa.

3.2. Quantification of released FMN

Treatment of the NQR pellet by LiOH released flavins which were analyzed by HPLC (Fig. 3). After 27 h at 0 °C in the dark, 7.0 ± 1.0 nmol riboflavin 5'-phosphate were extracted per mg Na^+-NQR, corresponding to a molar ratio of FMN to FAD of 1.7. The Na^+-NQR contained roughly 1 mol FAD [9,24], 1 mol riboflavin [9] and 2 mol of covalently bound flavins per mol of the complex (Table 1). The observed molar riboflavin/FAD ratio of 0.64 is caused by an incomplete occupancy of the riboflavin binding site in purified Na^+-NQR, as previously shown by reconstitution experiments [9]. In the case of FMN, the small discrepancy between the observed and expected content is probably caused by incomplete recovery of the hydrophobic NqrB and NqrC subunits after precipitation with TCA.

LiOH extracts were free of riboflavin, indicating that the phosphoester to the Thr but not to the ribityl side chain was cleaved, in accord with the proposed mechanism of β-elimination. The flavin released from NqrB and NqrC was authentic FMN with the phosphate group bound at the 5'-position of the ribityl. Since β-elimination under alkaline conditions is not expected to alter the position of phosphate on the ribityl side chain, the results indicate that a phosphodiester bridges the β-oxygen of Thr with the 5'-oxygen of ribityl. Yet these conclusions are only valid if no phosphate migration occurs after

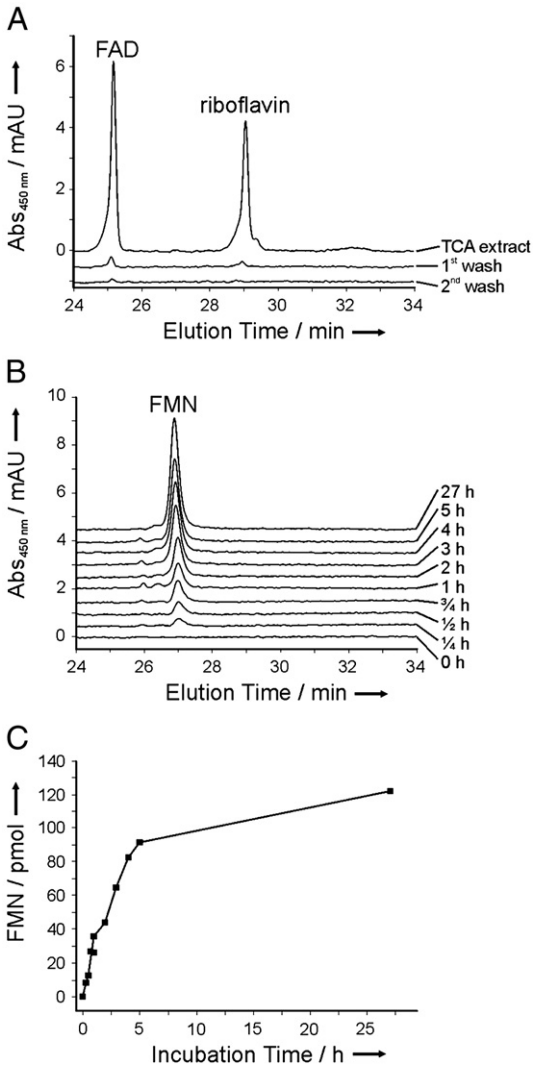

Fig. 3. Release of FMN from the Na^+-NQR under alkaline conditions. Na^+-NQR was precipitated with TCA, and the supernatant containing non-covalently bound flavins (FAD and riboflavin) was analyzed by HPLC. Washes of the precipitated Na^+-NQR were also analyzed (A). Precipitated Na^+-NQR was treated with LiOH to release covalently bound flavins. Aliquots of the supernatant were analyzed by HPLC at indicated time points (B), revealing a time-dependent release of FMN (C).

elimination of the riboflavin phosphate under alkaline conditions. This was confirmed for FMN, and for a 1:1 mixture of riboflavin 5'-phosphate (FMN) and riboflavin 4'-phosphate, which were treated with LiOH according to the protocol developed for the Na^+-NQR (Fig. 4). In contrast, treatment with 0.2 M HCl for 24 h at 56 °C converted riboflavin 4'-phosphate to FMN (Fig. 4), as described previously [11].

Table 1
Flavin cofactors in Na^+-NQR.

Flavin	Flavin:protein		Flavin:FAD
	[nmol mg^{-1}]	[mol mol^{-1}]	[mol mol^{-1}]
FAD	4.1 ± 0.2	0.88 ± 0.04	1.00
FMN	7.0 ± 1.0	1.50 ± 0.21	1.70
Riboflavin	2.6 ± 0.4	0.56 ± 0.08	0.64

Average from nine determinations (SD) with Na^+-NQR purified by affinity chromatography and gel filtration [24]. Content of Na^+-NQR was estimated by protein determination [10] assuming a molecular mass of 213 kDa.

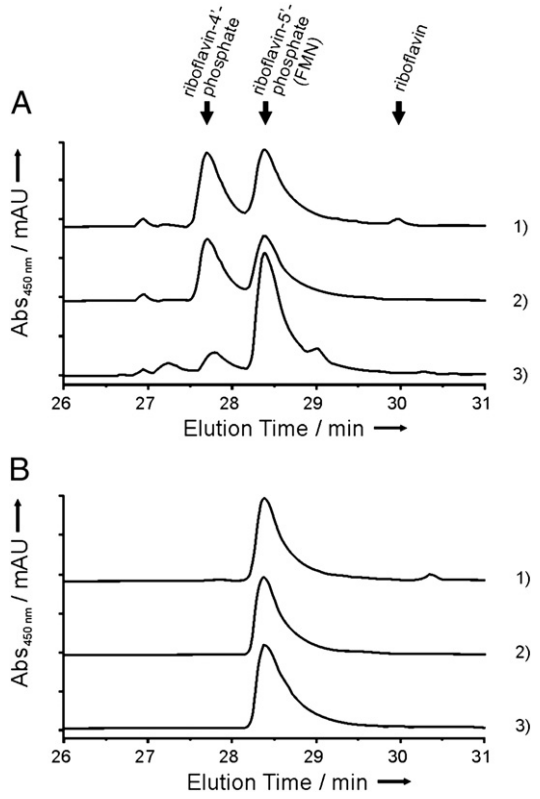

Fig. 4. Phosphate migration in riboflavin 4'-phosphate. HPLC profiles of a 1:1 mixture of FMN and riboflavin 4'-phosphate (A) and FMN (B). Trace 1, no addition; trace 2, with 0.5 M LiOH; trace 3, with 0.2 M HCl.

3.3. Localization of the FMN binding site by specific tagging of FMN-carrying threonine residues in the Na^+-NQR

The proposed mechanism of FMN cleavage from the Na^+-NQR (Fig. 5) predicts that the flavin moiety attached via a phosphodiester to the β-carbon of the Thr residue is eliminated under formation of a dehydro-2-aminobutyrate residue. To demonstrate the formation of this anticipated reaction product, the release of flavins by LiOH was performed in the presence of a nucleophile (β- mercaptoethanol) to

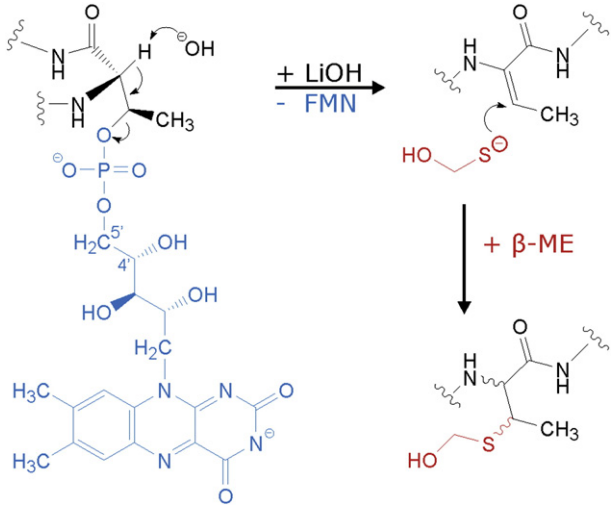

Fig. 5. FMN release from the Na^+-NQR by β-elimination followed by Michael addition. β-Elimination of FMN results in formation of dehydro-2-aminobutyrate residues. The dehydro-2-aminobutyrate residue readily reacts with a nucleophile (β-mercaptoethanol; β-ME) by Michael addition.

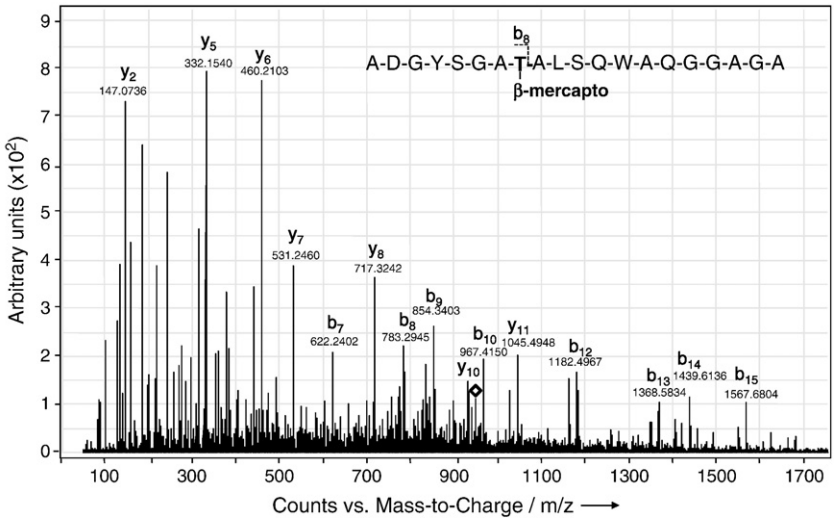

Fig. 6. Mass determination of a proteolytic peptide from NqrB modified with β-mercaptoethanol. The collision-induced dissociation product ion spectrum of the elastase-generated peptide ADGYSGATALSQWAQGGAGA (precursor $m/z = 949.9174$, $z = 2^+$) modified at position 8 (corresponding to Thr236) is shown. Individual b and y-type fragment ions are labeled. The diamond marks the precursor ion.

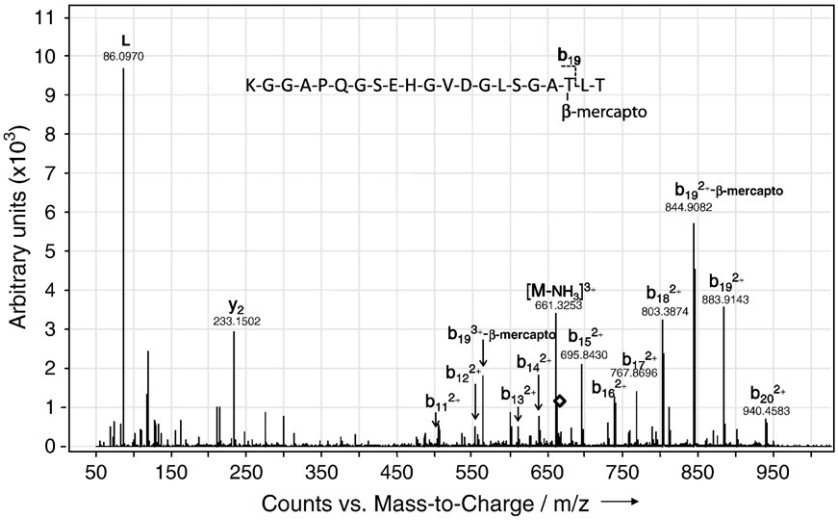

Fig. 7. Mass determination of a proteolytic fragment of NqrC modified with β-mercaptoethanol. The collision-induced dissociation product ion spectrum of the elastase-generated peptide KGGAPQGSEHGVDGLSGATLT (precursor $m/z = 666.9931$, $z = 3^+$) modified at position 19 (corresponding to Thr225) is shown. Individual b and y-type fragment ions are labeled. The diamond marks the precursor ion. Neutral loss of β-mercaptoethanol is observed from the fragment ions b_{19} and b_{20}.

capture dehydro-2-aminobutyrate residues in a Michael addition reaction. Subsequently, the masses of proteolytic fragments of Nqr subunits were determined. To increase the yield of peptide fragments, the solubilized holo-Na^+-NQR complex rather than precipitated subunits were used as source. LC-MS/MS analysis of elastase digests of NqrB and NqrC after β-elimination/Michael addition revealed high sequence coverage of 57% and 77%, respectively. Peptides containing Thr residues, which were modified by β-mercaptoethanol, were exclusively found in the expected region of NqrB (NqrB-Thr236; Fig. 6) and NqrC (NqrC-Thr225; Fig. 7) (Table 2). No other Thr or Ser residues were modified.

4. Conclusion

The study demonstrates that the phosphate group bridging the threonine residue in the Na^+-NQR with riboflavin is attached at the 5'-position of the ribityl chain of the flavin. We describe the mechanism of elimination of the covalently attached FMN under alkaline conditions and quantify the amount of FMN in the Na^+-NQR. To localize the FMN binding sites in the Na^+-NQR, the elimination of FMN is performed in the presence of β-mercaptoethanol, resulting in specific modification of FMN-carrying threonine residues. A combination of

Table 2
ESI-QTOF MS analysis of β-ME-modified peptides after β-elimination/Michael addition.

Sequence no.	Sequence	Score	Δm [ppm]
$229–248_{NqrB}$	ADGYSGAT*ALSQWAQGGAGA	86	−1
$206–227_{NqrC}$	VKGGAPQGSEHGVDGLSGAT*LT	49	7
$207–227_{NqrC}$	KGGAPQGSEHGVDGLSGAT*LT	97	7

Elastase-generated peptides of NqrB and NqrC after LiOH treatment in presence of β-ME were analyzed by ESI-QTOF MS for the presence of β-ME-modified Thr or Ser residues. Candidate peptides were identified using MASCOT with the designated β-ME modification. A total of three modified peptides were identified. MASCOT peptide scores and differences (errors) between the experimental and calculated masses in ppm are given. The asterisk indicates β-ME modification sites.

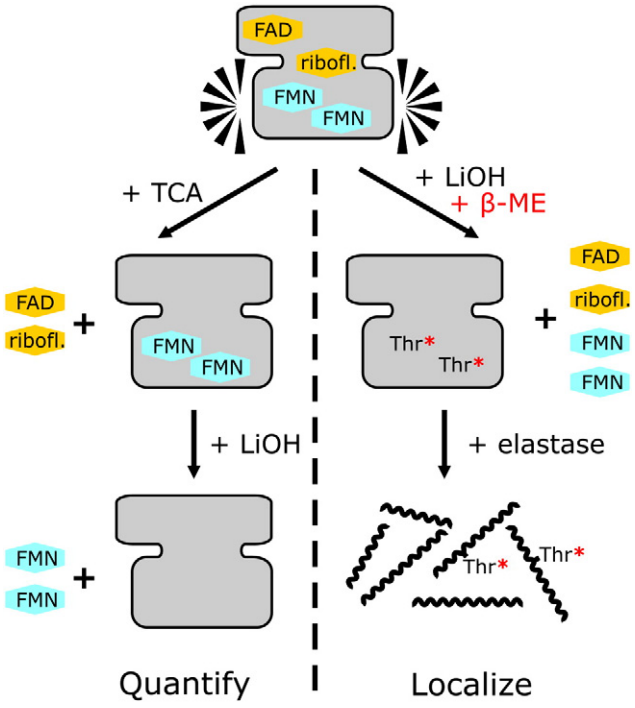

Fig. 8. Experimental strategies for quantification or localization of phosphodiester-linked flavins. Top, Na^+-NQR solubilized with DDM (black triangles) contains covalently attached FMNs, and non-covalently bound FAD and riboflavin. Left, denaturation and complete removal of the non-covalently bound FAD and riboflavin precedes the release of phosphodiester-linked flavins by β-elimination with LiOH and quantification by HPLC. Right, β-elimination and Michael addition with β-mercaptoethanol (β-ME) followed by proteolysis with elastase allows identification of Thr residues that carried the phosphodiester-linked FMNs in subunits NqrB and NqrC by MS analysis.

these strategies (Fig. 8) allows quantification of the full set of flavin cofactors in the Na^+-NQR and related membrane protein complexes.

Acknowledgments

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